Title: The influence of diazepam on cocaine and amphetamine regulated transcript (CART) peptides in the telencephalon of zebrafish

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Abstract:

Although the role of cocaine and amphetamine regulated transcript (CART) in energy metabolism is well established, recent studies have shown its involvement in processing of anxiety. Diazepam is commonly used to treat anxiety in humans. Herein we investigate the response of the endogenous CART immunoreactive system in the telencephalon following treatment with this agent. Zebrafish has emerged as a useful alternative to screen a range of activities including anxiety. We have studied the change in distribution pattern of CART in the telencephalon of zebrafish following diazepam administration. No changes were observed in the entopeduncular nucleus (EN), central (Dc), dorsal (Dd) and medial (Dm) zones of the dorsal telencephalic area. The population of CART immunoreactive fibres was greater in the lateral zone of the dorsal telencephalic area (Dl) and preoptic area (POA) but smaller in the dorsal zone of the ventral telencephalic area (Vd). We suggest that the CART system in these areas may be involved in the regulation of anxiety-like behaviour in zebrafish.

Keywords: CART, diazepam, Zebrafish (Danio rerio), anxiety, immunocytochemistry

1. Introduction:

Cocaine and amphetamine regulated transcript (CART), a neuropeptide with 116 amino acid residues, was first discovered in the hypothalamus of sheep by Speiss et al. (1981). The expression of the neuropeptide in the brain was up regulated in rats following the administration of cocaine or amphetamine, and therefore the name was conferred on the peptide (Douglass et al., 1995).

Since 1995, many variants and derived fragments of CART were reported from different species. Two alternatively spliced variants are seen in rats and mice. They differ in length by 13 amino acid residues and are called long and short variants (rl and rs in rats). However only one, the short variant, is expressed in humans (Kuhar et al., 2000). The CART peptide is processed downstream in different tissues and a large number of CART fragments were detected in the rat brain. Active fragments like rlCART55-102, rlCART62-102, rsCART42-89 and rsCART49-89 were detected in the brain and pituitary gland, while rsCART1-89 and rsCART10-89 were found in the adrenal glands of rat (Thim et al., 1999).

Among mammals, CART has been studied in rats, mice, sheep, humans, monkeys and voles (Kuhar and Dall Vechia, 1999; Hunter et al., 2005). The distribution pattern of CART was quite uniform across the different species studied. In the rat brain, CART is abundantly expressed in the hypothalamic nuclei like the arcuate and paraventricular nuclei. CART has also been detected in the olfactory bulb, the nucleus accumbens, the amygdala, the anterior and posterior pituitary, the parabrachial nucleus, the nucleus of the solitary tract, the, the dorsal horns of the spinal cord, the intermediolateral cell column of the spinal cord and some dorsal root ganglion cells (Koylu et al., 1998; Kuhar and Dall Vechia 1999).

The role of CART in the regulation of energy metabolism is well established. Intracerebroventricular injection of CART inhibited feeding behaviour in rats, whereas injecting an antibody against CART stimulated feeding (Lambert et al., 1998). When injected into the paraventricular nucleus, CART inhibited feeding induced by neuropeptide Y, an orexigenic peptide (Wang et al., 2000). Starved rats showed significantly lower levels of CART expression in the arcuate nucleus and lateral hypothalamus (Kristensen et al., 1998). CART expression in vagal neurons increased on injection of cholecystokinin, a hunger-suppressing gastric hormone (de Lartigue et al., 2007). CART mutant mice gained weight faster than wild-type littermates (Asnicar et al., 2001).

While a specific receptor molecule for CART is yet to be discovered, Yermolaieva et al. (2001) showed that CART inhibited L-type Ca^{2+} channels in a dose-dependent manner. CART increased phosphorylation of extracellular signal regulated kinase (ERK) and cyclic-AMP response element binding protein (CREB). All of the above actions of CART are in turn inhibited by pertussis toxin (Yermolaieva et al., 2001; Sarkar et al., 2004; Lakatos et al., 2005). These results indicate that CART may act via an inhibitory G-protein coupled receptor (Rogge et al., 2008).

CART peptides have been detected in the brain of non-mammalian vertebrates like catfish and frog (Lazar et al., 2004; Singru et al., 2007; Kobayashi et al., 2008; Roubos et al., 2008). CART shows anorexigenic-like activity in these animals as well as in goldfish, cod and chicken (Volkoff and Peter, 2001; Tachibana et al 2003; Kehoe and Volkoff, 2008).

CART is also involved in energy-status detection and in preparation for spawning in catfish (Barsagade et al., 2010; Subhedar et al., 2011)

In addition to energy metabolism, CART peptides affect the regulation of pain, arousal, startle response, neuroendocrine hormone secretion and ethanol withdrawal in mice (Bannon et al., 2001; Yermolaieva et al., 2001; Smith et al., 2004; Dandekar et al., 2008). Recent studies suggest a role for CART in anxiety. Kask et al. (2000) showed that following injection of CART, mice spent less time in the open arms of an elevated plus maze test, and the response was found to be dose dependent. CART injections also reduced oxygen consumption and gastric emptying rates (Asakawa et al., 2001). Interestingly, rlCART55-102 induced anxiety-like behaviour in mice, but not rlCART62-102 (Chaki et al., 2003).

The aim of this study is to find out if the endogenous CART system is involved in mediating anxiety. Zebrafish (*Danio rerio*) was used as the experimental animal model. Zebrafish has emerged as a useful alternative to rodents in recent years and widely employed in pharmacological investigations (Parng et al., 2002; Langheinrich, 2003; Kari et al., 2007). Diazepam is a well-characterized agent used to reduce anxiety in clinical practice. It is known to bind to $GABA_A$ receptors and enhance the inhibitory effect of GABA (Riss et al., 2008). We may recall that diazepam reversed the anxiogenic effect of CART in mice and reduced anxiety-like behaviour in zebrafish (Chaki et al 2003; Bencan et al., 2009).

Previous studies have shown some functional relationships between regions of the telencephalon and certain behavioural processes. The medial zone of the dorsal telencephalic area (Dm) is involved in learning avoidance behaviour, similar to the amygdala, while the lateral zone (Dl) may regulate spatial and temporal memory (Portavella et al., 2004) The dorsal zone of the ventral telencephalic area (Vd) mediates avoidance behaviour downstream of the Dm, similar to the striatum (Lau et al., 2011). Cells of the preoptic area (POA) appear to mediate displays of dominance or subordination (Koyama et al., 1984). In the present study, we treated zebrafish with diazepam and the response of the CART immunoreactive cells and fibers in the various above-mentioned components was investigated.

Acronyms:

Dc: Central zone of the dorsal telencephalic area.

Dd: Dorsal zone of the dorsal telencephalic area.

Dm: Medial zone of the dorsal telencephalic area.

Dl: Lateral zone of the dorsal telencephalic area.

EN: Entopeduncular Nucleus.

POA: Preoptic area.

Vd: Dorsal zone of the ventral telencephalic area.

Vs: Supracommissural zone of the ventral telencephalic area.

Vv: Ventral zone of the ventral telencephalic area.

2. Materials & methods:

2.1 Animals:
Sexually mature zebrafish of both sexes were used for the present study. Fish were housed in 3 litre tanks. A 14h light/10h dark cycle was maintained. Animals were fed twice daily with freshly hatched brine shrimp and once each day with complete adult zebrafish food (Ziegler). Water temperature was maintained at 28˚C (+/-1˚C) for the duration of the experiment. Dissolved oxygen was between 5-7 mg/L, while the pH was 6.5-7.7. Activated carbon filters and UV sterilizers (110mJ/cm^2) were used to ensure water quality; articulate matter was smaller than 50 µm. Hardness was between 50-200mg/L of $CaCO_3$. Conductivity of the water was maintained between 450 and 1000 µS (Zebrafish stand-alone system manual, Aquatic Habitats)

2.2 Diazepam administration and dissection:
The diazepam treatment was given at approximately 150-180 minutes after the scheduled feeding at 6:30 pm. This time schedule was followed to ensure uniformity across the fish population with reference to energy status and diurnal rhythms. The fish were immersed for a period of 3 mins in the solution of diazepam in distilled water (1.5mg/L). After the treatment, the fish were transferred to distilled water without diazepam for 5 mins. A similar protocol and dose was used by Bencan et al. (2009) to study the effect of diazepam on anxiety-like behaviour in zebrafish.

Thereafter the fish were anaesthetized with 2-phenoxyethanol (1:2000). The skull was opened from the dorsal side and the fish was fixed in 20 ml of ice-cold Bouin's fixative (75% saturated picric acid, 20% formaldehyde, 5% glacial acetic acid). After fixation at 4°C for 18-20 hours, the brain was dissected out, cryoprotected in 25% sucrose overnight at 4°C, embedded in OCT medium (Tissue-tek™, Sakura-Finetek). While control fish were treated similarly, no diazepam was used.

2.3 Immunocytochemistry:
Embedded brains were sectioned serially in transverse plane at 10 µm thickness using a cryostat (Leica). Serial sections were stretched on a poly-L-lysine coated slide. The slides were rinsed in 0.1% PBSTx (0.1% TritonX-100 dissolved in phosphate buffer saline), blocked in 5% HIGS (5% heat-inactivated goat serum dissolved in 0.1%PBSTx) for 1 hour and incubated in monoclonal mouse anti-CART antibodies for 15 hours at 4˚C. Slides were then rinsed again in 0.1% PBSTx, blocked again in 5% HIGS for 1 hour and incubated in goat anti-mouse-Ig antibodies labelled with Alexa Fluor488 for 3 hours. The sections were rinsed in 0.1%PBSTx and mounted with fluorescence mounting medium (90% glycerol, 0.5% propyl gallate, 20mM Tris-Cl). Sections from the control and diazepam treated brain were processed together to maintain uniformity of staining conditions.

2.4 Morphometric Analysis:
The distribution of CART immunoreactive cells and fibres in the telencephalon and preoptic area of control fish was determined using Apotome microscope, AxioCam cameras and AxioVision software (version 4.8) (Zeiss). For the relative quantitative analysis of the CART immunoreactivity, images of the area Dc, Dd, Dl, Dm, EN, Vd and POA from the brain of the diazepam treated were collected. 5 images of each area from a fish, and a total of 30 images from six different brains were collected. All images were collected at 200x magnification. Images were in monochrome and saved as 16-bit in Tagged Image File (.tif) format. Similar images were collected from the brain sections of the control fish.

The area occupied by CART immunoreactive cells and fibres in each area of the telencephalon was obtained from these images. A background subtraction (2x median grey value of the area of interest) was performed on each image to remove artefacts and non-specific fluorescence. The images were then converted to binary (8-bit, black and white only) and fluorescence in each region was measured by calculating the percentage of area occupied by white spots in the image. Measurements were taken from a strictly defined area in each region to maintain uniformity.

A two-tailed Student's T-Test was performed to compare the percentage area data from 30 images of sections from control and from diazepam treated fish.

2.5 Counting CART expressing cells in EN:
CART expressing neurons in the EN were counted using the Apotome microscope at 200X magnification. Care was taken to ensure that no sections of the telencephalon were lost while cutting. The start and end of the EN were carefully noted. Cells were counted from both sides of the brain from sections that contained the EN. 10 control and 10 diazepam treated fish were used for this experiment.

To avoid over-counting of cells the following correction factor was applied:

$N_V=(N_A*D)/(T+D)$

[N_V = Corrected number of nuclei over whole volume, N_A = Raw number of nuclei summed over all sections, T = Thickness of the section (T=10 μm), D = Mean diameter of nuclei (D = 6.72 μm)] (Abercrombie, 1946)

A two-tailed Student's T-Test was performed to compare the cell count data from the brains of 10 control and 10 diazepam treated fish.

3. Result:

3.1 Distribution of CART in the telencephalon and preoptic area of the zebrafish brain:

In the ventrolateral region of the telencephalon, the EN appears as a conspicuous group that shows a large number of CART immunoreactive neurons. These neurons are present along the pial surface and extend about 40 μm into the sub-pial region. CART immunoreactive fibres that probably arise from the CART neurons in the EN can be traced in mediodorsal direction (Fig 1 **A**). A few solitary CART immunoreactive neurons were also seen in the Vv.

CART immunoreactive fibres were largely confined to the dorsal areas. Bright immunofluorescent fibers were seen in the Dc, Dd, Dl, Dm and POA. While long solitary fibres were seen the ventral telencephalic area, dense clusters of fibers were evident in the Vd (Fig 1 **B-G**).

3.2 Effect of diazepam administration on CART expression:

The number of CART expressing cells in the EN of the diazepam-treated fish was similar to that of the control (Fig. 2 A-D) and no significant differences were noticed ($p = 0.97$). Similarly, in the Dc, Dd, and Dm regions, the percentage of area occupied by CART immunoreactive fibres was almost identical in both groups of fish (Figs. 3-5) (**Dc** $p = 0.495$; **Dd** $p = 0.388$; **Dm** $p = 0.462$). However, there were significant differences between control and diazepam treated fish in the area occupied by CART immunoreactive fibres in Dl, Vd and POA (Figs. 6-8). Diazepam treated fish showed a larger percentage area occupied by CART immunoreactive fibres in Dl ($p << 0.001$) and POA ($p = 0.007$) than control fish. On the other hand, the percentage area occupied by CART immunoreactive fibres in Vd of diazepam treated fish was smaller than that of the control fish ($p = 0.021$).

A few CART immunoreactive fibers were detected in the Vv and Vs of the control as well as treated fish and these were not included in the current study.

4. Discussion:

Studies on the behavioural effects of CART have consistently shown that the peptide might serve as an endogenous anxiogenic agent. Rats injected with CART showed greater anxiety-like behaviour in elevated plus maze (EPM) and social interaction tests (Kask et al., 2000). CART injected mice showed reduced oxygen consumption and gastric emptying rates, signifying greater anxiety (Asakawa et al., 2001). In EPM and social interaction tests, CART induced anxiety was reversed by diazepam in rats (Chaki et al., 2003). However, there is no information on the response of the endogenous CART system to diazepam treatment. The present study aims at determining the changes, if any, in the CART system following treatment with diazepam.

Previous studies have screened the anxiolytic effects of diazepam five minutes after the administration (Bencan et al., 2009) and the same interval was employed in the present study.

The EN is a major CART containing group in the telencephalon of teleosts and is believed to play a role in energy metabolism (Singru et al., 2007; Subhedar et al., 2011). We observed no difference in the population of CART expressing neurons in the EN of control and fish-treated with diazepam for 3 mins. The data suggests that the CART containing neurons of the EN may not be sensitive to the diazepam, and perhaps have no role in processing anxiety. However, it would be necessary to test higher doses of diazepam, and longer treatment time frames, to conclusively resolve the issue.

Similarly there was no difference between the percentage area of CART fibres in the Dc, Dd and Dm regions in the telencephalon of the control and diazepam treated groups. The Dm is believed to be the homologue of the amygdala and the Dc appears to be a collection of cells and fibres from different pallial regions (Butler, 2000). CART expression in these areas may also have no role in anxiety.

However, a different pattern of response was obtained in Dl, Vd and POA following diazepam treatment. In the treated fish, Dl and POA showed a higher percentage of area occupied by CART expressing fibres than that in the control fish. The Dl is reported to be involved in the regulation of spatial and temporal memory, while POA is a major neuroendocrine centre and regulates different behaviours including sexual arousal and displays of dominance /subordination (Portavella et al., 2004; Forlano and Bass, 2010; Bandoh et al., 2011). While the neuronal origin of these fibres is not known, we speculate that the change may be causally linked to the diazepam treatment. It would be interesting to find out if CART fibre terminals in Dl and POA are also involved in processing anxiety-like information.

Diazepam treatment seems to have reduced the percentage area occupied by the CART fibres as compared to that in the control fish. The Vd is believed to be a homologue of the mammalian striatum (Forlano and Bass, 2010). While we do not know the underlying reasons, we suggest that the reduction might be due to release of CART from the fibre terminals in Vd. The possibility that this area may also process anxiety related information is suggested.

$GABA_A$ receptors are ubiquitous in the telencephalon and hypothalamus of zebrafish (Kim et al., 2003). Within the telencephalon, the largest concentrations appear to be in the Dm,

Vd and Vv (Davey et al., 2010). Since diazepam acts only on $GABA_A$ receptors, it is likely that changes in GABAergic neurons in the aforementioned regions affect processes downstream, leading to the observed changes in CART distribution.

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Figure legends:

(Arrows indicate neurons and arrowheads indicate CART immunoreactive fibres.)

Figure 1: CART expression in the rostral telencephalon. **A,** Schematic transverse section through the rostral telencephalon of zebrafish showing major CART containing areas like the entopeduncular nucleus (EN), central zone of the dorsal telencephalic area (Dc) dorsal zone of the dorsal telencephalic area (Dd), medial zone of the dorsal telencephalic area (Dm), lateral zone of the dorsal telencephalic area (Dl) and dorsal zone of the ventral telencephalic area (Vd). **B-G,** Transverse sections through the telencephalon showing CART cells (arrows) and fibres of the entopeduncular nucleus (**B**), and fibres in the Dc (**C**), Dd (**D**), Dm (**E**), Dl (**F**), Vd (**G**).

Figure 2: CART expression in the caudal telencephalon. **A** Schematic transverse section through the caudal telencephalon of zebrafish showing CART expressing regions like EN, Dc, Dm, and preoptic area (POA). **B**, Section through POA showing CART immunoreactive fibres.

Figure 3: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing cells in entopeduncular nucleus (EN). The population of neurons per fish (**C**) did not differ significantly across the two groups (N= 10 fish; Control: 324.65; Dzp treated: 323.57; Student's T Test p= 0.969911)

Figure 4: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing fibres of Dc. The percentage area of CART expression was measured over 50000 μm^2 of Dc. The percentage area did not differ significantly between the two groups (N= 6; Control: 2.992; Dzp treated: 2.500; Student's T Test p= 0.495046).

Figure 5: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing fibres of the Dd. The percentage area of CART expression was measured over 15000 μm^2 of Dd (**C**) The percentage area did not differ significantly between the two groups (N= 6; Control: 3.258; Dzp treated: 3.709; Student's T Test p = 0.388407).

Figure 6: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing fibres of the Dm. The percentage area of CART expression (**C**) was measured over 45000 μm^2 of Dm from each section. The percentage area did not differ significantly between the two groups (N= 6; Control: 1.683; Dzp treated: 1.586; Student's T Test p= 0.462615).

Figure 7: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing fibres of the Dl. The percentage area of CART expression (**C**) was measured over 36000 μm^2 of Dl from each section. The difference in the percentage area of CART expression between the two groups was highly significant. The percentage area of CART expression was 63.88% higher in diazepam treated fish as compared to that in the control (N=6; Control: 1.069; Dzp treated: 1.752; Student's T Test p= 0.00001097)

Figure 8: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing fibres of the Vd. The percentage area of CART

expression (**C**) was measured over 40000 μm^2 of Vd from each section. The difference in the percentage area of CART expression between the two groups was significant. The percentage area of CART expression was 29.39% lower in diazepam treated fish as compared to that in the control. (N=6; Control: 0.616; Dzp treated: 0.435; Student's T Test p= 0.020916).

Figure 9: Sections through the telencephalon of control (**A**) and diazepam treated (**B**) zebrafish showing CART expressing fibres of the POA. The percentage area of CART expression (**C**) was measured over 20000 μm^2 of POA in every section. The difference in the percentage area of CART expression between the two groups was highly significant. The percentage area of CART expression was 68.37% higher in diazepam treated fish as compared to that in the control (N=6; Control: 0.724; Dzp treated: 1.219; Student's T Test p= 0.006720).

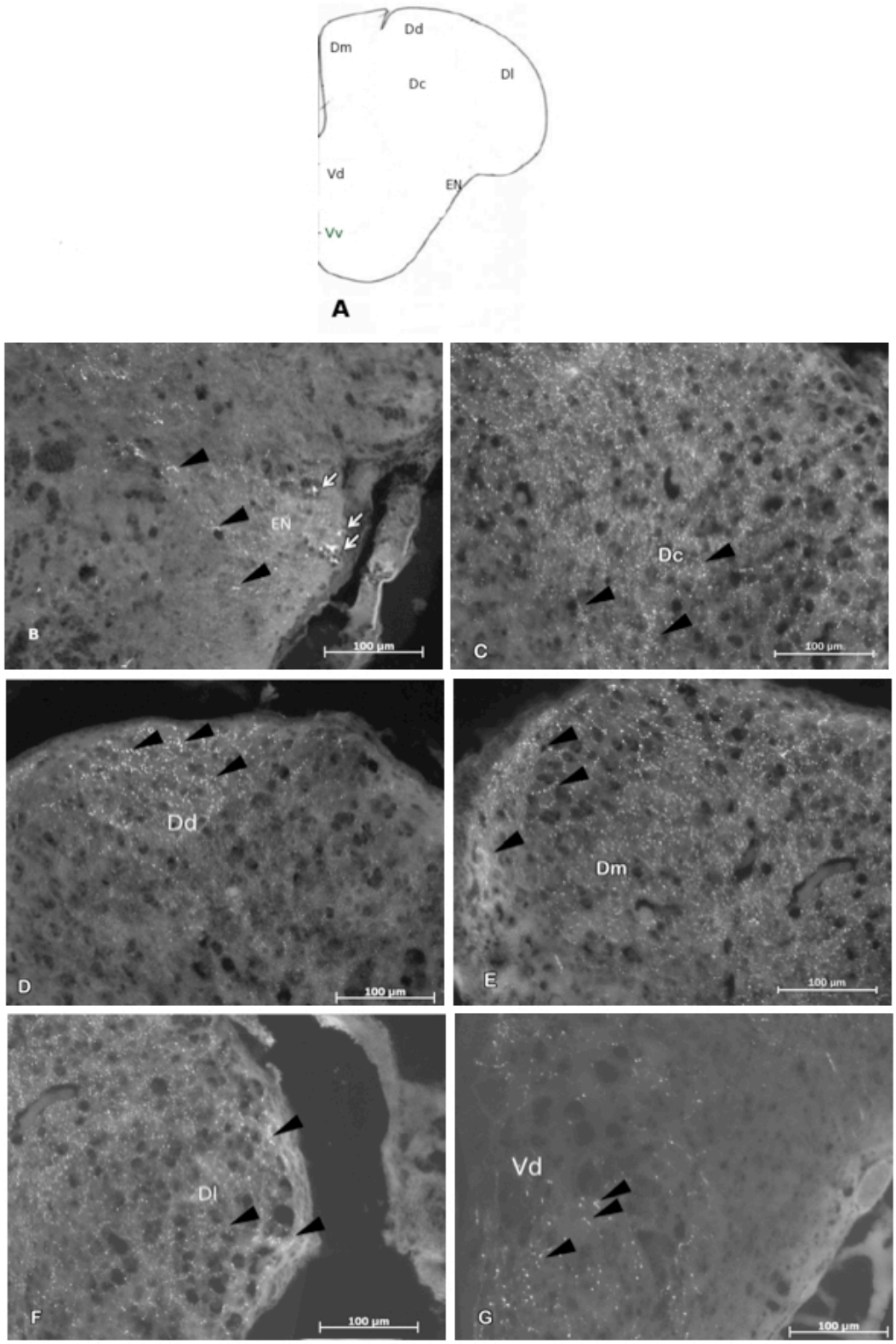

Fig 1 (A, B, C, D, E, F, G)

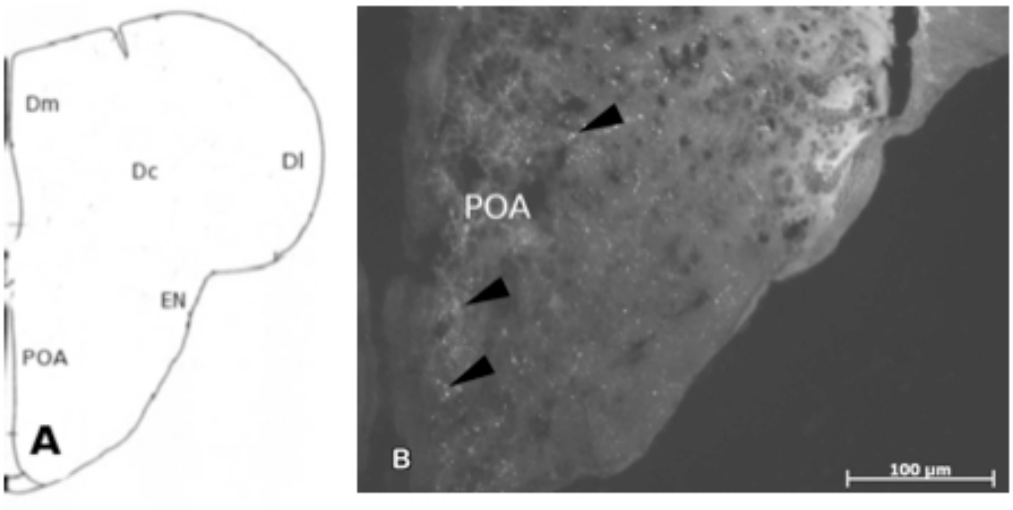

Fig 2 (A, B)

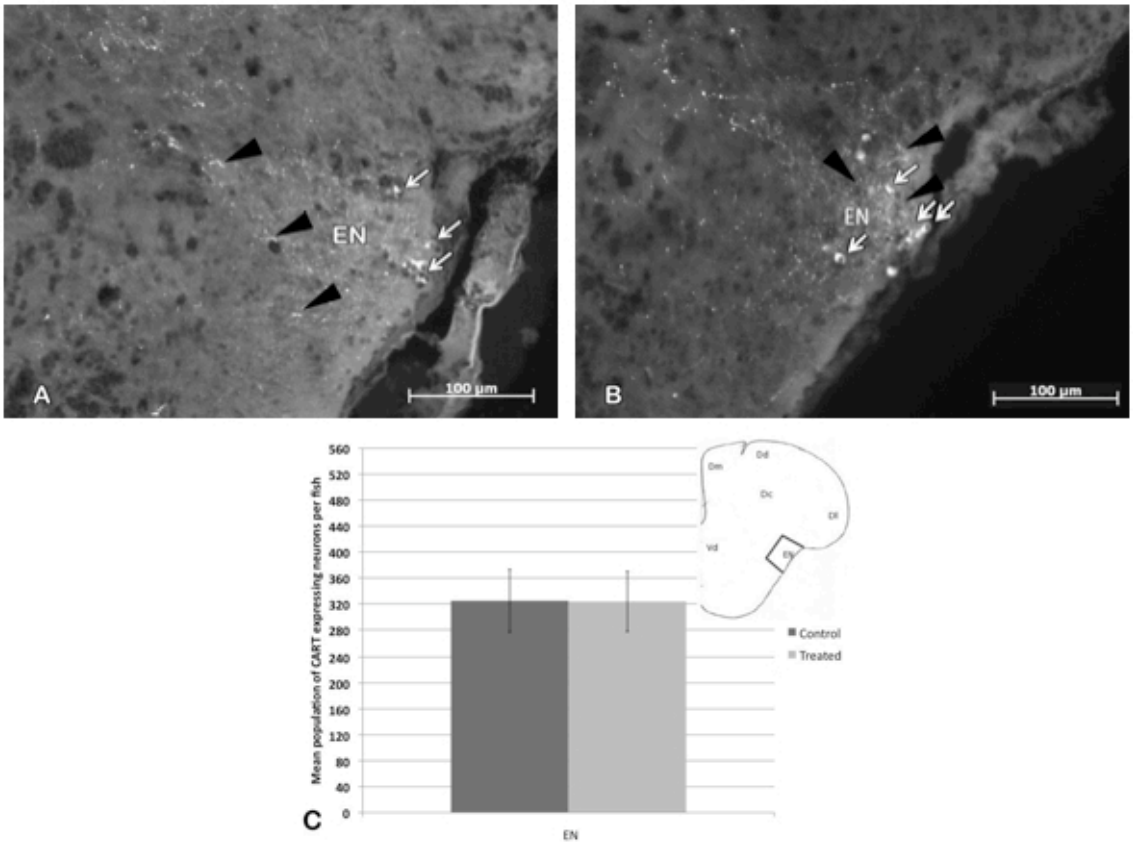

Fig 3 (A, B, C)

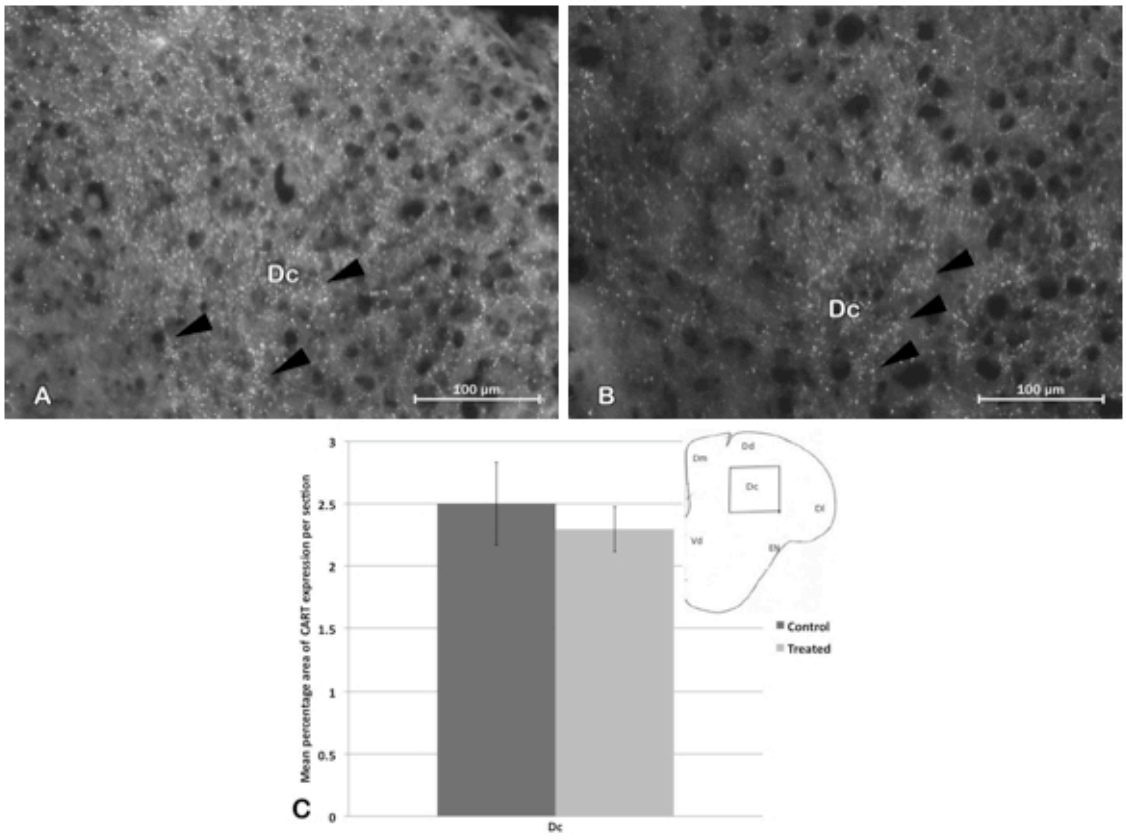

Fig 4 (A, B, C)

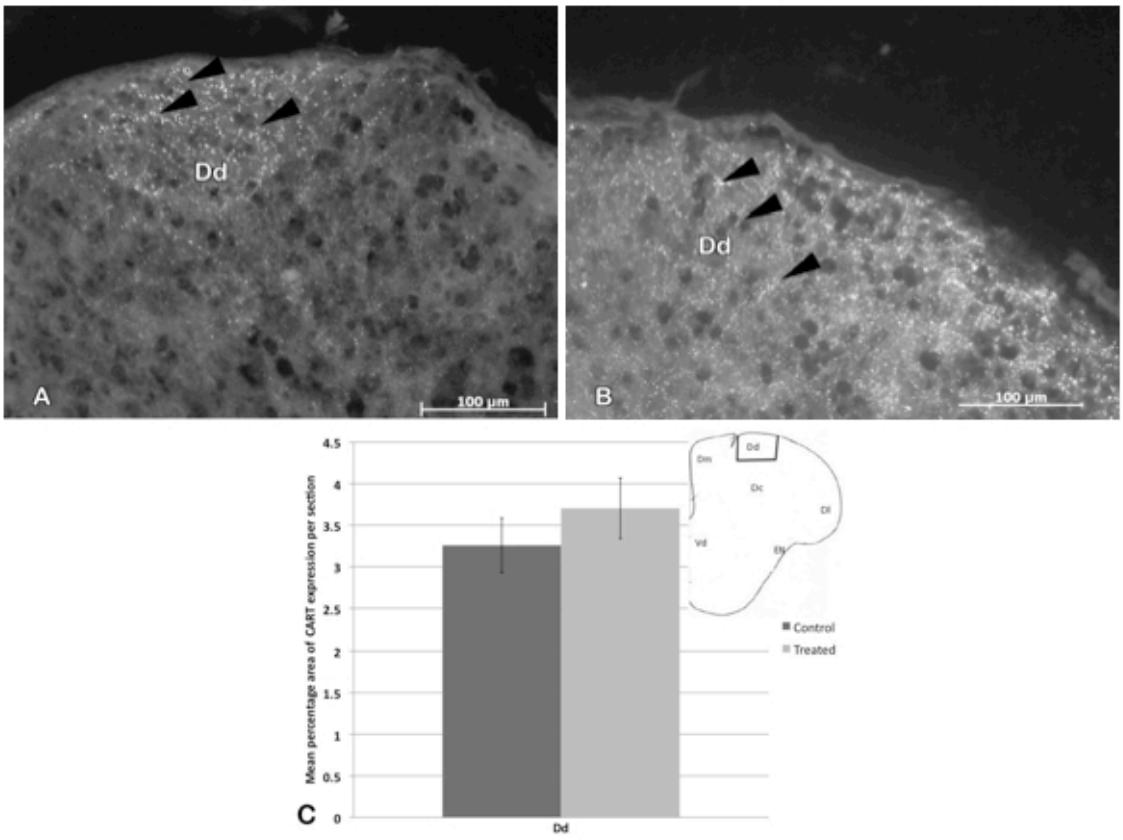

Fig 5 (A, B, C)

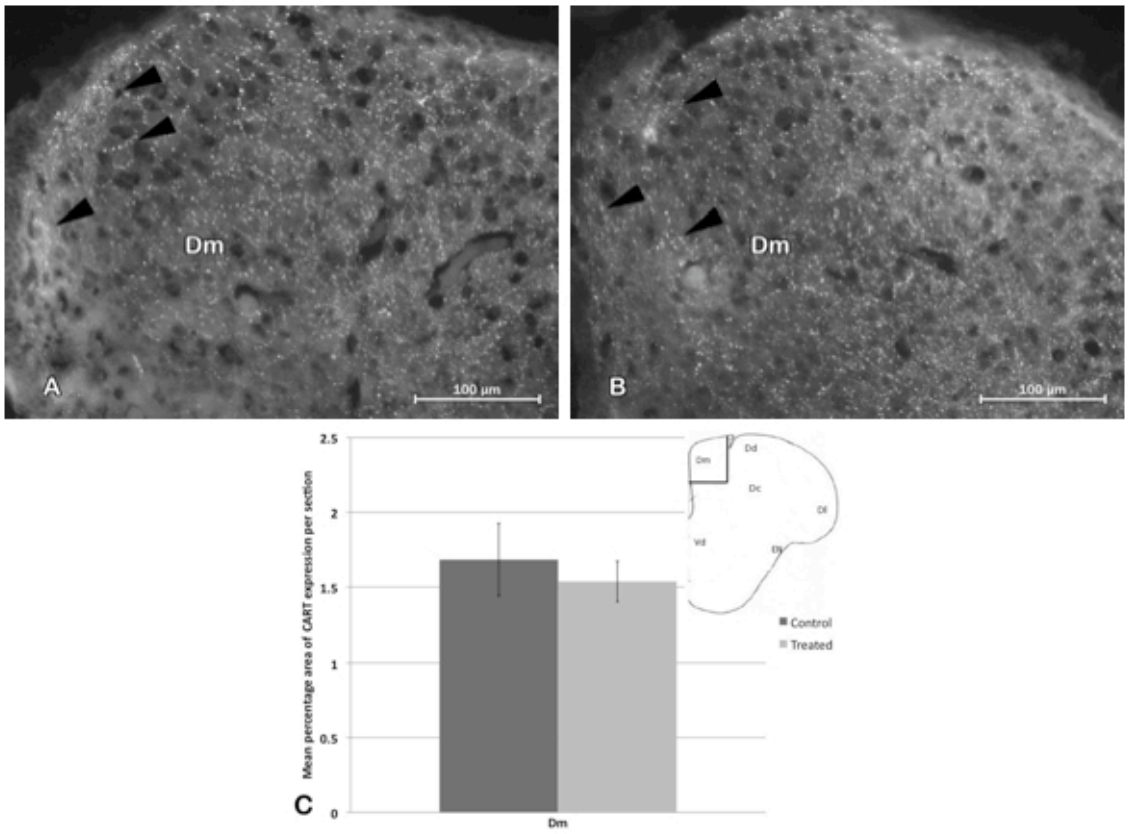

Fig 6 (A, B, C)

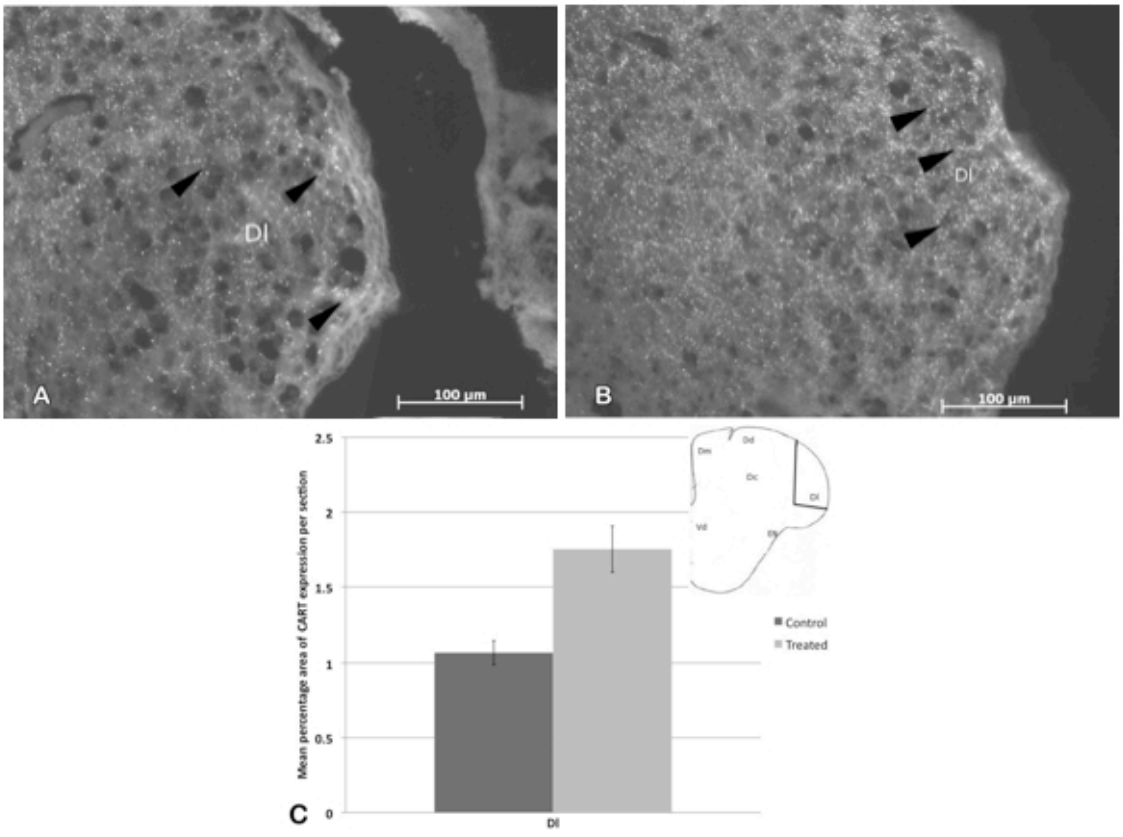

Fig 7 (A, B, C)

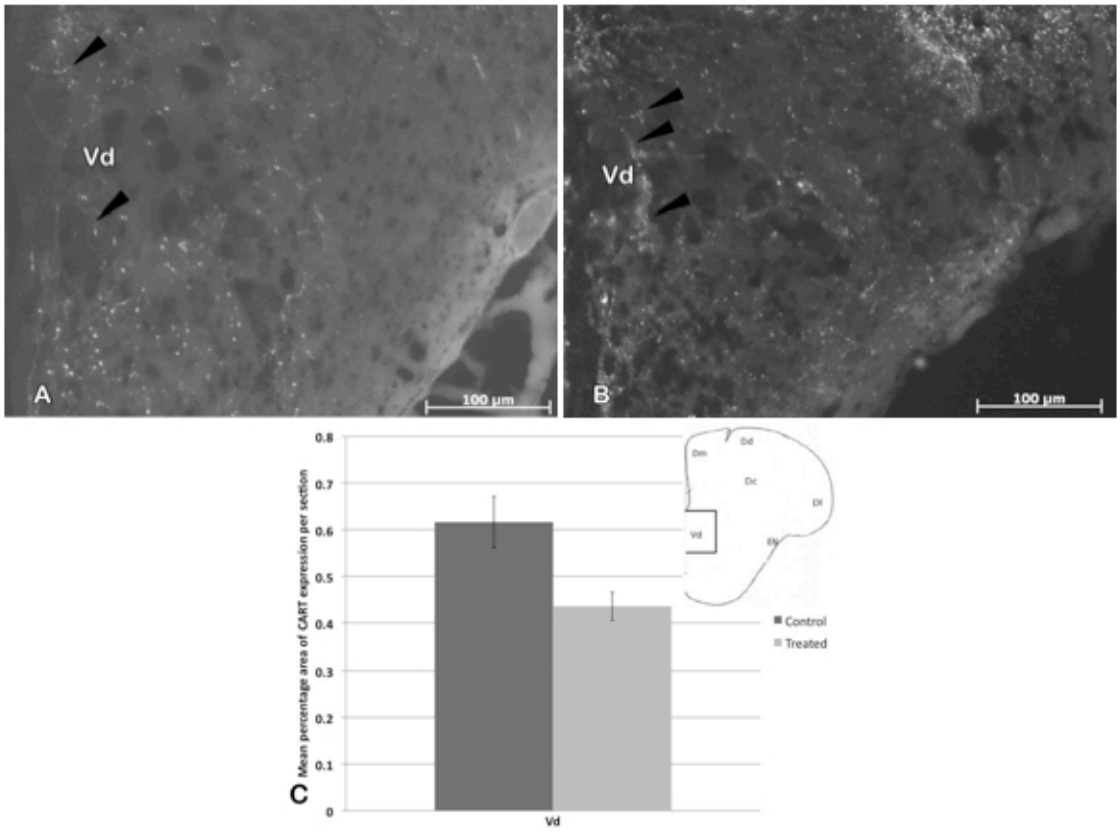

Fig 8 (A, B, C)

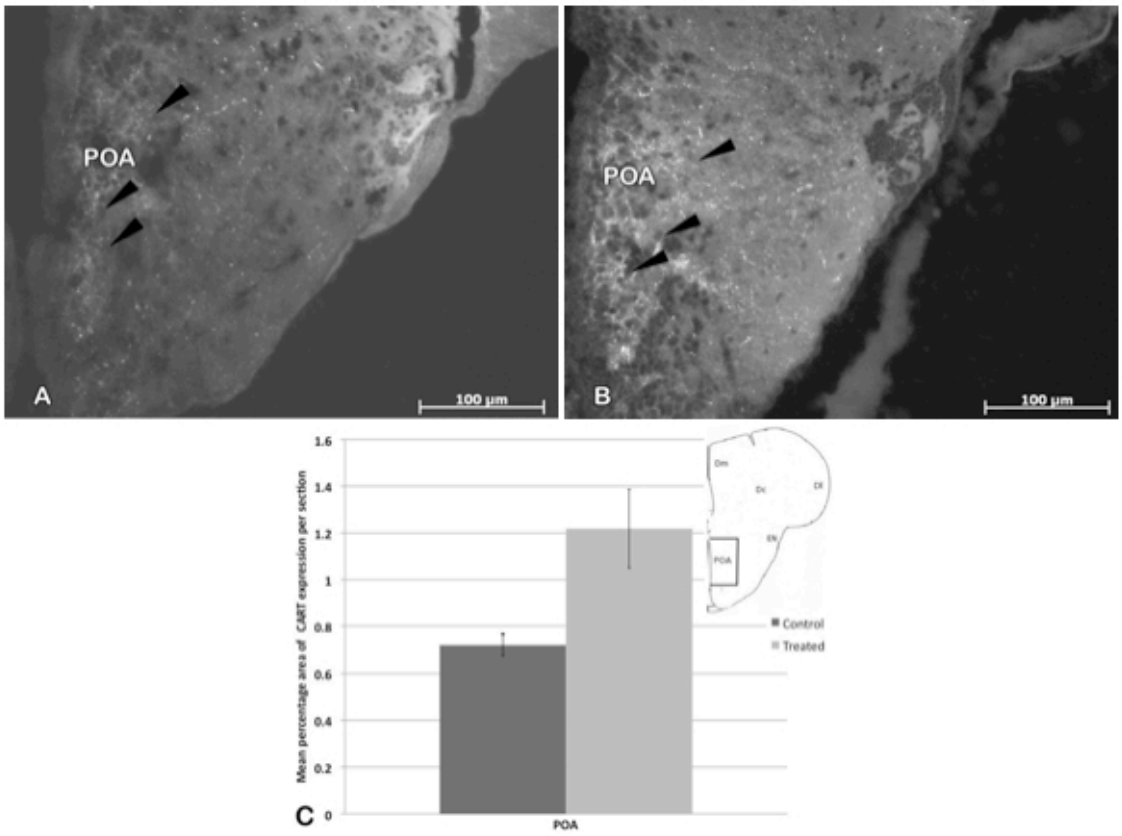

Fig 9 (A, B, C)